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Decreased proliferation of HepG2 liver cancer cells in vitro and exhibited proteomic changes in vivo in subjects with metabolic syndrome and metabolic dysfunction-associated steatotic liver disease who performed four-week dawn-to-dusk dry fasting

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Abstract

Background Four-week dawn-to-dusk dry fasting (DDDF) was previously shown to have a potent anti-inflammatory effect and induce an anti-tumorigenic proteome in the serum and peripheral blood mononuclear cells in subjects without cancer. The study goal was to determine if serum obtained from these subjects without cancer who underwent 4-week DDDF has an anti-tumorigenic effect.

Methods HepG2 cells were treated with serum collected from four individuals with metabolic syndrome and metabolic dysfunction-associated steatotic liver disease (MASLD) and four healthy individuals who performed 4-week DDDF. The objective was to assess cell proliferation/viability in HepG2 cells treated with non-fasted and dry-fasted serum and determine proteomic changes in human serum. We comparatively performed 3-[4,5-dimethylthiazol-2-yl]-2,5-diphenyltetrazolium bromide (MTT) cell proliferation assay and untargeted proteomic analysis using nano ultra-high performance liquid chromatography coupled with tandem mass spectrometry.

Results Serum collected from 3 out of 4 subjects with metabolic syndrome and MASLD at the end of 4-week DDDF (dry-fasted serum/V2) significantly reduced proliferation/viability in HepG2 cells compared with the serum collected before 4-week DDDF (non-fasted serum/V1). A similar reduction effect on cell proliferation was not observed when HepG2 cells were treated with dry-fasted serum collected from healthy subjects. In addition to the in vitro changes observed, the following circulating gene protein products (GP) demonstrated significant increases or decreases in subjects with metabolic syndrome and MASLD after a 4-week DDDF regimen, compared with their GP levels before

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the 4-week DDDF: CD248 molecule (mean log2 fold = 8.124, $P = 0.001$), dipeptidyl peptidase 4 (mean log2 fold = 0.937, $P = 0.027$), lymphatic vessel endothelial hyaluronan receptor 1 (mean log2 fold = 1.054, $P = 0.029$), LDL receptor related protein 1 (mean log2 fold = 1.401, $P = 0.031$), and beta-2-microglobulin (mean log2 fold= -0.977, $P = 0.033$) at the end of 4-week DDDF compared with the GP levels before 4-week DDDF.

Conclusion This study demonstrated that dry-fasted serum collected from subjects with metabolic syndrome and MASLD decreased HepG2 cell proliferation in vitro and showed that proteomic changes occurred in vivo. These findings suggest that DDDF may be an effective intervention for inducing proteomic responses that could assist in the prevention and adjunct treatment of cancers associated with metabolic syndrome.

Keywords Dry fasting, Intermittent fasting, Dawn-to-dusk dry fasting, Diurnal fasting, Daytime fasting, Human serum, Liver cancer, Hepatoblastoma, Human serum proteome

Introduction

A global epidemic of metabolic syndrome and metabolic dysfunction-associated steatotic liver disease (MASLD) is tightly related to obesity [1, 2]. Metabolic syndrome, a significant risk factor for developing systemic inflammation, endothelial dysfunction, and MASLD, is diagnosed when three or more of the five adverse clinical features are present, including increased waist circumference, insulin resistance, elevated blood pressure, high blood triglyceride, and low high-density lipoprotein levels [3–6]. Importantly, subjects with metabolic syndrome and MASLD are at significant risk for developing malignancies, including hepatocellular carcinoma and several other cancers (e.g., pancreatic and colon cancer) [7, 8]. Therefore, to confer anti-tumorigenic effects, an ideal therapeutic regimen for subjects with metabolic syndrome and MASLD should improve metabolic parameters, induce a robust anti-inflammatory effect, and correct intracellular molecular aberrancies.

Intermittent fasting has been shown to have an anti-inflammatory and anti-tumorigenic effect [9–13]. In the murine osteosarcoma model, food deprivation during the activity period was shown to have the most robust anti-tumor effect compared with food deprivation during the inactivity period and ad libitum feeding [13]. The results were linked to improved host control over the tumor, alterations in the tumor's circadian rhythms, or a combination of both [13]. Water fasting cycles of 48 to 60 h delay tumor progression and increase sensitivity to chemotherapy drugs in murine melanoma, neuroblastoma, and breast cancer models [9]. This is consistent with the Warburg effect that represents metabolic stress and the reprogramming of protein translation that could be fatal to malignant cells [14–16]. In humans, a similar anticancer effect may be achieved through a shorter fasting cycle if fasting is done dry (without eating or drinking) from dawn to dusk over several consecutive days [10–12].

Our previous studies on non-cancerous individuals suggest that dry fasting from dawn to dusk can promote an anti-inflammatory and anti-tumor proteome response, increasing tumor-suppressing proteins and decreasing those linked to tumor promotion [10–12]. Therefore, to validate the anti-tumorigenic effect of dawn-to-dusk dry fasting (DDDF) observed in subjects without cancer, we hypothesized that serum collected at the end of 4-week DDDF (i.e., proteome-conditioned serum by 4-week DDDF/dry-fasted serum) could reduce proliferation/viability of HepG2 liver cancer cells compared with serum collected before 4-week DDDF (non-fasted serum) (in vitro experiment) and induce proteomic changes in the same subjects' serum at the end of 4-week DDDF compared with serum collected before 4-week DDDF (in vivo experiment).

Several features of DDDF make it a superior alternative to prolonged water/wet fasting regimens (water and zero-calorie drinks are allowed during fasting) to achieve anticancer effect: DDDF is compliant with zeitgeber modulated (external cues) circadian rhythm because mealtimes occur precisely at dawn and dusk immediately before and after fasting window. Brief pulses of light at dawn and dusk are sufficient to entrain the central clock in the suprachiasmatic nucleus of the anterior hypothalamus and establish a 24-hour circadian rhythm, while mealtimes strongly entrain peripheral clocks [17, 18]. It is thought that peripheral clocks are more vulnerable to modulation by food-related stimuli, such that a relative desynchronization between the central and peripheral clocks might increase the risk of metabolic dysfunction [19]. Therefore, regularly taking meals at dawn and dusk would also align the peripheral circadian clock phase with the central clock phase entrained to local dawn and dusk, potentially mitigating the risks of metabolic syndrome [11, 12]. The DDDF model stands in contrast to skipping breakfast routine that has been employed in several time-restricted eating (TRE) regimens and having late-night eating that disrupts the circadian regulation of metabolism [20–25]. The energy intake is imbalanced by irregular cues that misalign the peripheral clocks with the central clock, normally entrained to local dawn and dusk cues [19, 20–25].

Dry fasting is reported to protect against skeletal muscle and vital body weight loss that can occur with water/

wet fasting due to the degradation of proteins as alternative energy sources when glycogen stores are depleted during fasting [26]. In fact, a randomized clinical trial of a TRE regimen that allowed study subjects to have water, black coffee and tea during the fasting window led to substantial lean mass loss [25]. Water/wet fasting should not be considered an actual fasting regimen but a water/zero calorie drink-based diet [26, 27]. Drinking during fasting likely inhibits vasopressin secretion, independent of plasma osmolality status, which also appears sufficient to indirectly misalign peripheral clocks [28]. Vasopressin is a powerful stimulant of lipolysis and beta-oxidation of fatty acids [29]. The inhibition of vasopressin leads to the inhibition of adrenocorticotropic hormone, resulting in the subsequent suppression of epinephrine release [30, 31]. Epinephrine depletion has been shown to increase protein degradation during fasting, likely due to the inactivation of lipoprotein lipase; in turn, the fatty acids cannot be utilized as an energy source alternative to glucose during fasting [29, 32, 33]. Taken altogether, in theory, the act of drinking (water or any liquid) during water/wet fasting will shift energy production from glucose toward utilizing amino acids from skeletal muscle instead of fatty acids as a fuel substrate and result in muscle mass and vital body weight loss [26, 28].

DDDF is straightforward to comply with as the fasting window is from dawn to dusk if it is approached as part of a ritual [34], and this stands in contrast to 48 h to 60 h of continuous starvation.

With adequate hydration during the non-fasting window, DDDF was shown to have an excellent safety profile in various populations, including healthy individuals, subjects with increased body mass index, metabolic syndrome and MASLD, and renal transplant patients [10–12, 35–39]. Dry fasting is anticipated to induce metabolic water production (metabolism of 100 g of fat produces over 100 g of endogenous water) [26, 40–42]. An observational study involving 34 participants practicing daytime dry fasting found that both plasma and 24-hour urine osmolality were within the normal physiological range during the fasting period [38]. Dehydration during DDDF can be readily prevented with adequate hydration during non-fasting window, nutrition education, and avoiding conditions that can lead to dehydration during both non-fasting and fasting windows.

The practice of shorter fasting windows in the DDDF, particularly during the winter months, can minimize the likelihood of overeating during non-fasting periods and reduce the risk of refeeding syndrome. This potentially serious condition that can arise following extended periods of fasting [43].

To determine the anti-tumorigenic effect of 4-week DDDF, we treated HepG2 cells with serum collected from subjects with metabolic syndrome and MASLD and healthy subjects (control group) who performed 4-week DDDF. HepG2 cell line was initially reported to be isolated from a 15-year-old male with hepatocellular carcinoma [44, 45]. However, a later publication reported that the HepG2 cell line was derived from an epithelial hepatoblastoma, not a hepatocellular carcinoma [46]. Our primary aim was to determine whether serum collected at the end of 4-week DDDF (i.e., serum conditioned by 4-week DDDF/dry-fasted serum) can reduce proliferation/viability of HepG2 cells compared with serum collected before 4-week DDDF (non-fasted serum) (in vitro experiment). Our secondary aim was to determine proteomic changes in the same subjects' serum at the end of 4-week DDDF compared with serum collected before 4-week DDDF (in vivo experiment).

Methods

Human serum collection

Prior to conducting this study, approval from the Institutional Review Board for Human Subject Research for Baylor College of Medicine and Affiliated Hospitals (Protocol number H-31612) was obtained. The inclusion and exclusion criteria, study design, and protocol H-31612 procedures to collect data and specimens from subjects with metabolic syndrome and MASLD, and healthy subjects who performed 4-week DDDF were previously reported [10, 11].

For the treatment of HepG2 cells, we used stored serum specimens from four subjects (three men and one woman, mean age 67 years [SD=6]) with metabolic syndrome and MASLD (Fig. 1) and four healthy male subjects (mean age=32 years [SD=6]) who practiced 4-week DDDF (Fig. 1). For both groups, we used the serum collected before 4-week DDDF (non-fasted serum) and at the end of 4-week DDDF (dry-fasted serum).

Determination of HepG2 cell proliferation/viability: 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) assay

HepG2 cells were plated into flat-bottomed 96-well plates at a density of 5000 cells per well. Cells were incubated in a full-growth medium at 37 °C for 6 h until they were adherent to the plate. Media containing 20% V/V fetal bovine serum (FBS) (control) or human serum samples were added to each well. After 24 h, 15 µl of Dye Solution (CellTiter 96® Non-Radioactive Cell Proliferation Assay #G4000, Promega, Madison, WI, USA) was added to each well, and the plates were incubated at 37 °C in a humidified 5% CO2 incubator for 4 h. 100 µl of Solubilization Solution/Stop mix was added to each well, and the plates were incubated in a CO2 incubator at 37 °C overnight. The plates were shaken on a horizontal shaker for 30 s to allow for complete dissolution, and then the absorbance of the plates was recorded at 570 nm wavelength using a

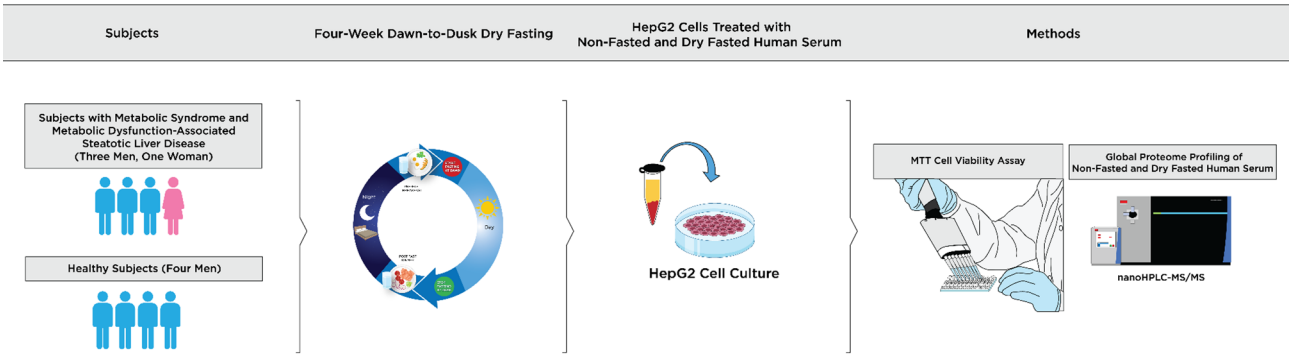

Fig. 1 Subjects with metabolic syndrome and metabolic dysfunction-associated steatotic liver disease (MASLD) and healthy subjects fasted (strict dry fasting without food or drink intake) from dawn to dusk for over 14 h daily for 4 weeks. The serum was collected before 4-week DDDF (non-fasted serum) and at the end of 4-week DDDF (dry-fasted serum). After HepG2 cells were treated with non-fasted and dry-fasted serum, cell proliferation/viability in HepG2 cells was assessed by 3-[4,5-dimethylthiazol-2-yl]−2,5 diphenyltetrazolium bromide (MTT) cell proliferation/viability assay. An untargeted proteomic analysis was performed using nano ultra-high performance liquid chromatography-tandem mass spectrometry to determine the changes in proteins in non-fasted and dry-fasted serum collected from the same subjects

96-well plate reader. All the experiments were performed in triplicate.

Human serum proteomic analysis

The proteomic profiling of serum using a mass spectrometer was conducted following established protocols [11]. Briefly, the serum samples were thawed at 37 °C, and 10 µl were collected for analysis. The 14 most abundant serum proteins were depleted using a commercial kit (Thermo Scientific, Cat# A36370). The remaining proteins were digested with trypsin on an S-Trap column (ProtiFi, NY), and the resulting peptides were vacuum-dried. These peptides were then fractionated into two pools using the high pH STAGE method [47] and analyzed with Thermo Scientific EASY-nLC 1000 coupled Orbitrap Fusion™ Tribrid™ Mass Spectrometer (Thermo Fisher Scientific). Peptide data were converted to quantifiable gene protein products (GPs) using the label-free, intensity-based absolute quantification (iBAQ) method and normalized to the final quantitative value (iFOT, defined as iBAQ of the individual identified protein divided by the total iBAQ of all identified proteins within one experiment) using proprietary software, as previously detailed [48].

Statistical analysis

For statistical analyses, Microsoft® Excel® for Microsoft 365 MSO (Version 2504 Build 16.0.18730.20186) 64-bit software program (Microsoft, Redmond, WA, USA) and SAS software, Version 9.4 TS Level 1M7 X64_10PRO platform (SAS Institute Inc., Cary, NC, USA) [49] were used.

To determine statistically significant differences in HepG2 cell proliferation/viability at the end of 4-week DDDF compared with the HepG2 cell proliferation/viability before 4-week DDDF, independent two-tailed student's t-tests (P value < 0.05) were performed.

The statistical analysis of proteomics was performed on three subjects with metabolic syndrome and MASLD, and three healthy subjects after removing one outlier subject from both groups. For analysis, log-converted intensity-based fraction of total iFOT values were used [10–12]. The GP level at the end of 4-week DDDF compared with the GP level before 4-week DDDF was considered differentially expressed if the GP level showed greater than or equal to 1.5-fold (log2 fold greater than or equal to 0.585) mean paired change with a P-value of < 0.05 [10–12].

We also used Omics Playground [50] for Prize-Collecting Steiner Forest (PCSF) algorithm to identify high-confidence subnetworks of highly correlated and differentially expressed proteins [51], with the goal of uncovering potential "driver" proteins that function as network hubs. The analysis was performed using the STRING protein-protein interaction network as a template.

Results

MTT cell proliferation/viability assays

MTT assay in HepG2 cells treated with non-fasted and dry-fasted serum collected from subjects with metabolic syndrome and MASLD

Compared to HepG2 cells treated with human serum collected before 4-week DDDF (non-fasted serum), HepG2 cells that were treated with human serum collected at the end of 4-week DDDF (dry-fasted serum) showed significantly reduced cell proliferation/viability in 3 out of 4 subjects with metabolic syndrome and MASLD (Fig. 2). In subject 1, on the first assay, mean HepG2 cell viability (% of fetal bovine serum control) for V1 vs. V2 was 204.50 (SD = 3.92) vs. 174.62 (SD = 12.00), $P = 0.015$, on the second assay, mean HepG2 cell viability for V1 vs. V2 was 231.38 (SD = 23.13) vs. 172.01 (SD = 9.69), $P = 0.015$;

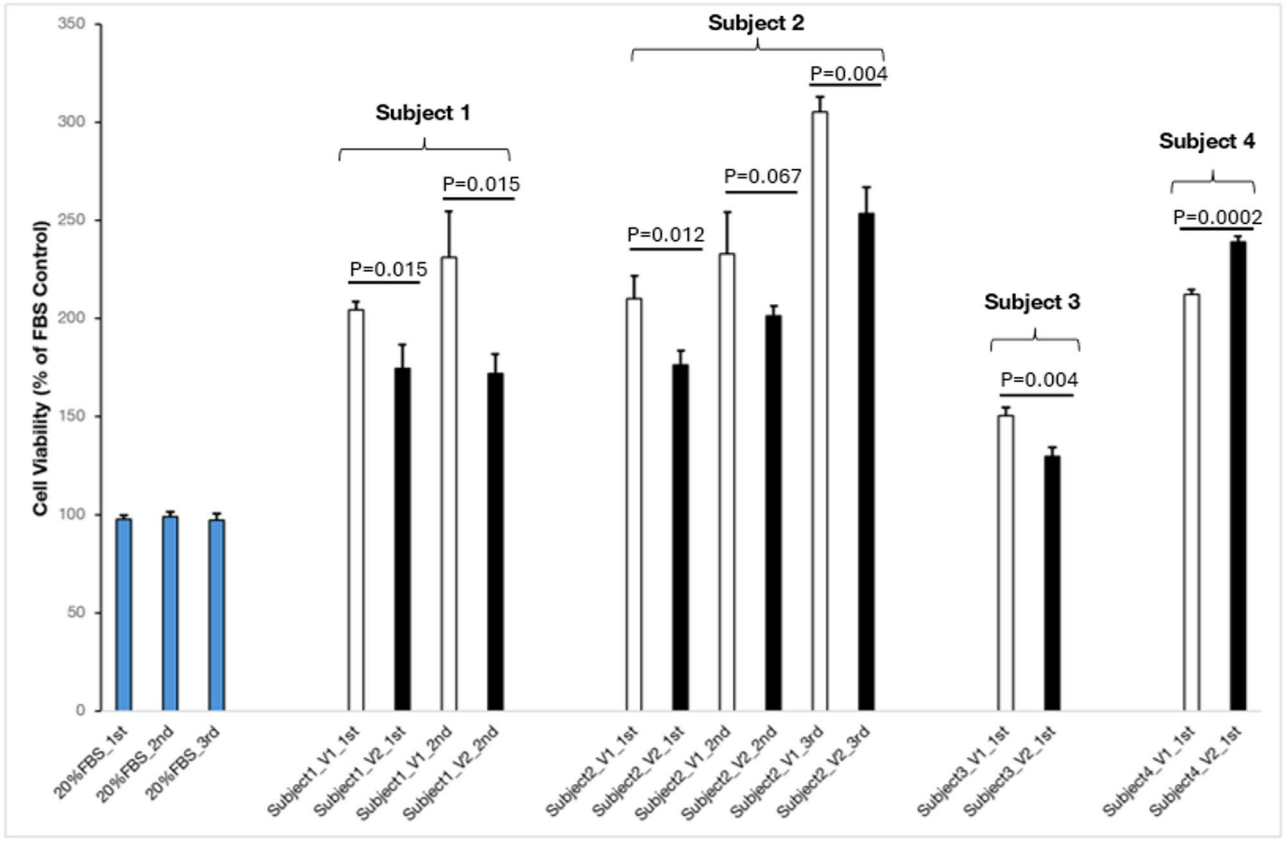

Fig. 2 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) cell proliferation/viability assay on HepG2 cells treated with non-fasted (V1) and dry-fasted (V2) serum collected from subjects with metabolic syndrome and metabolic dysfunction-associated steatotic liver disease (MASLD) who fasted from dawn to dusk for 4 weeks (4-week DDDF). Compared to HepG2 liver tumor cells treated with human serum collected before 4-week DDDF (non-fasted serum), HepG2 cells that were treated with human serum collected at the end of 4-week DDDF (dry-fasted serum) showed significantly reduced cell proliferation in 3 out of the 4 subjects with metabolic syndrome and MASLD. A similar reduction in cell proliferation/viability was not observed in subject 4 with metabolic syndrome and MASLD. The effect of fetal bovine serum (FBS) on HepG2 cell proliferation/viability was much lower than that of non-fasted and dry-fasted human serum on HepG2 cell proliferation/viability

in subject 2, on the first assay, mean HepG2 cell viability for V1 vs. V2 was 210.23 (SD = 11.39) vs. 176.14 (SD = 7.29), $P = 0.012$, on the second assay, mean HepG2 cell viability for V1 vs. V2 was 232.88 (SD = 21.29) vs. 201.28 (SD = 5.08), $P = 0.067$, on the third assay, mean HepG2 cell viability for V1 vs. V2 was 305.16 (SD = 7.83) vs. 253.76 (SD = 13.31), $P = 0.004$; in subject 3, on the first assay, mean HepG2 cell viability for V1 vs. V2 was 150.64 (SD = 3.78) vs. 130.00 (SD = 4.43), $P = 0.004$. A similar reduction in cell viability was not observed in subject 4 with metabolic syndrome and MASLD: on the first assay, mean HepG2 cell viability for V1 vs. V2 was 212.47 (SD = 2.10) vs. 239.14 (SD = 2.61), $P = 0.0002$.

MTT assay in HepG2 cells treated with non-fasted and dry-fasted serum collected from healthy subjects

Compared to HepG2 cells treated with human serum collected before 4-week DDDF (non-fasted serum), HepG2 cells that were treated with human serum collected at the end of 4-week DDDF (dry-fasted serum) showed no significant decrease in cell proliferation/viability in healthy subjects (Fig. 3). In subject 1, on the first assay, V1 vs. V2 mean HepG2 cell viability (% of fetal bovine serum control) was 190.14 (SD = 2.69) vs. 222.95 (SD = 5.30), $P = 0.001$, on the second assay, V1 vs. V2 mean HepG2 cell viability was 311.94 (SD = 4.55) vs. 326.24 (SD = 20.80), $P = 0.309$; in subject 2, on the first assay, V1 vs. V2 mean HepG2 cell viability was 306.88 (SD = 16.68) vs. 292.69 (SD = 5.04), $P = 0.231$, in subject 3, on the first assay, V1 vs. V2 mean HepG2 cell viability was 292.58 (SD = 14.95) vs. 257.96 (SD = 16.02), $P = 0.052$, in subject 4, on the first assay, V1 vs. V2 mean HepG2 cell viability was 247.40 (SD = 24.27) vs. 254.63 (SD = 11.93), $P = 0.668$.

MTT assay in HepG2 cells treated with FBS

The effect of FBS on HepG2 cell proliferation/viability was much lower than that of non-fasted and dry-fasted human serum on HepG2 cell proliferation/viability (Figs. 2 and 3). The mean cell viability in HepG2 cell culture treated with FBS was 97.72 (SD = 1.98) (first assay),

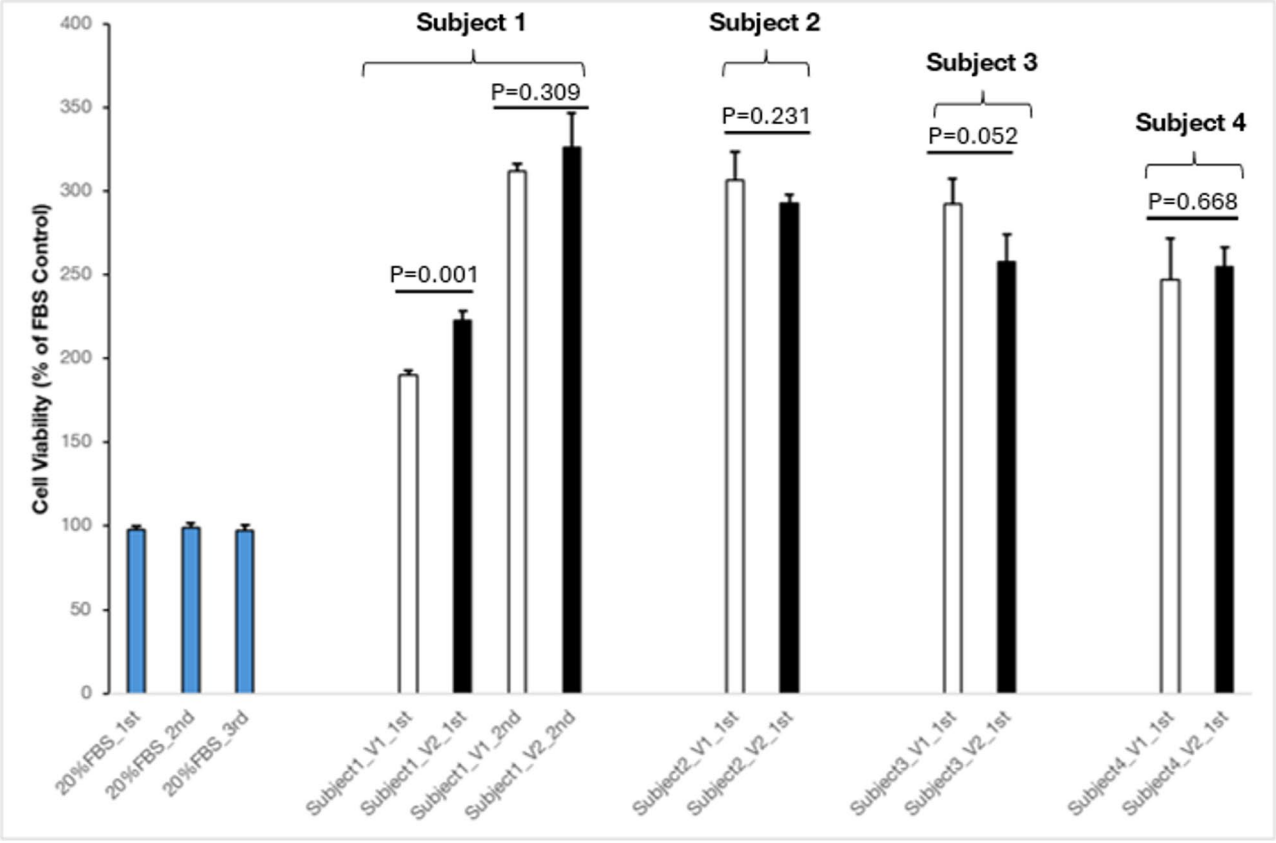

Fig. 3 3-(4,5-dimethylthiazol-2-yl)−2,5-diphenyltetrazolium bromide (MTT) cell proliferation/viability assay on HepG2 cells treated with non-fasted (V1) and dry fasted (V2) serum collected from healthy subjects who fasted from dawn to dusk for 4 weeks (4-week DDDF). There was no significant decrease in cell proliferation in HepG2 cells that were treated with human serum collected at the end of 4-week DDDF (dry fasted serum) compared to HepG2 cells treated with human serum collected before 4-week DDDF (non-fasted serum). The effect of fetal bovine serum (FBS) on HepG2 cell proliferation/viability was much lower than that of non-fasted and dry-fasted human serum on HepG2 cell proliferation/viability

Table 1 Differentially expressed gene protein products (GP) in serum collected from subjects with metabolic syndrome and metabolic-dysfunction associated steatotic liver (MASLD) who performed 4-week dawn-to-dusk dry fasting (DDDF)

Gene Symbol	Gene ID	Gene Name	Average Paired Log2 Fold Change*	Paired *P* Value
GP Levels that Increased at the End of 4-Week DDDF Compared with the GP Levels Before 4-Week DDDF				
CD248	57124	CD248 Molecule	8.124	0.001
DPP4	1803	Dipeptidyl Peptidase 4	0.937	0.027
LYVE1	10894	Lymphatic Vessel Endothelial Hyaluronan Receptor 1	1.054	0.029
LRP1	4035	LDL Receptor-Related Protein 1	1.401	0.031
GP Levels that Decreased at the End of 4-Week DDDF Compared with the GP Levels Before 4-Week DDDF				
B2M	567	Beta-2-Microglobulin	−0.977	0.033

* A positive mean paired log2 fold change indicates an increase, and negative mean paired log2 fold change indicates a decrease in the levels

99.32 (SD = 2.22) (second assay), and 97.42 (SD = 3.18) (third assay).

Human serum proteomics

Serum proteomics in subjects with metabolic syndrome and MASLD

The GPs that had greater than or equal to 1.5-fold mean paired change and a P-value of < 0.05 at the end of 4-week DDDF compared with the GP levels before 4-week DDDF are included in Table 1. There was an average 279 fold increase in CD248 molecule (CD248) (mean log2 fold = 8.124, $P = 0.001$), 2 fold increase in dipeptidyl peptidase 4 (DPP4) (mean log2 fold = 0.937, $P = 0.027$), 2 fold increase in lymphatic vessel endothelial hyaluronan receptor 1 (LYVE1) (mean log2 fold = 1.054, $P = 0.029$), 3 fold increase in LDL receptor-related protein 1 (LRP1) (mean log2 fold = 1.401, $P = 0.031$) GP levels compared with the GP levels before 4-week DDDF. There was a significant decrease in beta-2-microglobulin (BM2) (mean log2 fold= −0.977, $P = 0.033$) GP levels at the end of 4-week DDDF compared with the levels before 4-week DDDF. We included peptide spectrum match (PSM) of

differentially expressed GPs in Additional file Table S1. The mean fold changes observed in all GPs are included in Additional file Table S2.

Serum proteomics in healthy subjects

The GPs that had greater than or equal to 1.5-fold mean paired change and a P-value of <0.05 at the end of 4-week DDDF compared with the GP levels before 4-week DDDF are included in Table 2. There was an average 2-fold increase in charged multivesicular body protein 4 A (CHMP4 A) (mean log2 fold = 0.661, $P = 0.045$) GP levels compared with the GP levels before 4-week DDDF. There was a significant decrease in follistatin like 4 (FSTL4) (mean log 2 fold= −9.016, $P = 0.0002$), cystatin A (CSTA) (mean log2 fold= −12.325, $P = 0.0003$), cathepsin C (CTSC) (mean log2 fold= −8.542, $P = 0.001$), lipocalin 2 (LCN2) (mean log2 fold= −11.271, $P = 0.002$), fatty acid binding protein 5 (FABP5) (mean log2 fold=−12.127, $P = 0.010$), profilin 1 (PFN1) (mean log2 fold=−12.865, $P = 0.012$), tyrosine 3-monooxygenase/tryptophan 5-monooxygenase activation protein epsilon (YWHAE) (mean log2 fold= −9.769, $P = 0.026$), CD109 molecule (CD109) (mean log2 fold= −1.321, $P = 0.039$), CD93 molecule (CD93) (mean log2 fold= −2.567, $P = 0.041$), filamin A (FLNA) (mean log2 fold= −10.331, $P = 0.0498$) at the end of 4-week DDDF compared with the GP levels before 4-week DDDF. We included PSM of differentially expressed GPs in Additional file Table S3. The mean fold changes observed in all GPs are included in Additional file Table S4.

Table 2 Differentially expressed gene protein products (GP) in serum collected from healthy subjects who performed 4-week dawn-to-dusk dry fasting (DDDF)

Gene Symbol	Gene ID	Gene Name	Average Paired Log2 Fold Change*	Paired *P* Value
GP Levels that Increased at the End of 4-Week DDDF Compared with the GP Levels Before 4-Week DDDF				
CHMP4A	29082	Charged Multivesicular Body Protein 4A	0.661	0.045
GP Levels that Decreased at the End of 4-Week DDDF Compared with the GP Levels Before 4-Week DDDF				
FSTL4	23105	Follistatin Like 4	−9.016	0.0002
CSTA	1475	Cystatin A	−12.325	0.0003
CTSC	1075	Cathepsin C	−8.542	0.001
LCN2	3934	Lipocalin 2	−11.271	0.002
FABP5	2171	Fatty Acid Binding Protein 5	−12.127	0.010
PFN1	5216	Profilin 1	−12.865	0.012
YWHAE	7531	Tyrosine 3-Monooxygenase/Tryptophan 5-Monooxygenase Activation Protein Epsilon	−9.769	0.026
CD109	135228	CD109 Molecule	−1.321	0.039
CD93	22918	CD93 Molecule	−2.567	0.041
FLNA	2316	Filamin A	−10.331	0.0498

* A positive mean paired log2 fold change indicates an increase, and negative mean paired log2 fold change indicates a decrease in the levels

Prize-Collecting Steiner Forest (PCSF) algorithm analysis

The analysis of the PCSF subnetwork showed that proteins associated with the EGFR and APOA1 pathways were generally decreased in serum samples collected from subjects with metabolic syndrome and MASLD at the end of 4-week DDDF compared with the serum collected before 4-week DDDF (Fig. 4A). In contrast, EGFR and APOA1 node protein levels were elevated in serum samples taken from healthy subjects at the end of 4-week DDDF compared with the serum collected before 4-week DDDF (Fig. 4B).

Discussion

We cultured HepG2 cells using non-fasted and dry-fasted human serum from two distinct groups of subjects and analyzed the serum proteome of the same individuals. This led to three critical observations: (1) Serum collected from most of the subjects with metabolic syndrome and MASLD at the end of 4-week DDDF significantly decreased cell proliferation/viability in HepG2 cells compared with the serum collected before 4-week DDDF; (2) serum collected from healthy subjects at the end of 4-week DDDF did not significantly decrease cell proliferation/viability in HepG2 cells; and (3) there was a differential gene expression (both increases and decreases) when comparing serum protein levels at the end of the 4-week DDDF with those measured before the 4-week DDDF in both groups.

The capability of dry-fasted serum collected from subjects with metabolic syndrome and MASLD to decrease cell proliferation in HepG2 liver tumor cell cultures could be due to the anti-tumorigenic proteome in the serum [11]. Our previous work showed that 4-week DDDF increased tumor-suppressor and DNA-repair proteins and decreased tumor-promoter proteins in the serum of subjects with metabolic syndrome [11]. We also showed that peripheral blood mononuclear cells (PBMC) collected from subjects with metabolic syndrome who performed 4-week DDDF showed an anti-inflammatory, anti-atherosclerotic, and anti-tumorigenic proteome [12]. Human serum conditioned by acute aerobic exercise was shown to have a similar anti-proliferative effect on LoVo human colon cancer cells [52]. A meta-analysis of 9 in vitro studies that evaluated the effect of exercise-conditioned human serum collected from 244 individuals showed that acute exercise-conditioned human serum significantly reduced proliferation in cancer cell lines, with the effect being more pronounced with acute high-intensity exercise-conditioned human serum [53]. These

findings indicate that DDDF, mealtimes, and exercise impact cancer behavior. Human serum conditioned by 4-week DDDF combined with acute high-intensity exercise may substantially reduce cancer cell proliferation compared with their individual effects alone. Further studies are needed to confirm this hypothesis.

We observed a significant reduction in cell proliferation in HepG2 cells treated with dry-fasted serum collected from subjects with metabolic syndrome and MASLD. However, no similar reduction was seen in these cells when treated with dry-fasted serum from healthy subjects. This suggests that the impact of DDDF on subjects with metabolic syndrome may be more pronounced compared with its impact on healthy individuals. Consequently, it is important to include more healthy subjects in the study to confirm this effect. Additionally, the lack of a significant reduction in the growth of HepG2 cells treated with dry-fasted serum from healthy subjects may be attributed to certain substances or factors present in their serum. Therefore, a greater volume of dry-fasted serum may be necessary to mitigate the influence of these factors. Several reports showed that human serum had substances that control the mode (dense clumps vs. loose migratory structure) and cell growth rate in tissue cultures [54–57]. Different effects of human serum were observed by others in cell cultures based on distinct populations (healthy vs. pregnant vs. cancer) on tissue cultures [54–57]. Whereas normal human serum had a cell-proliferating effect, human serum collected from individuals with neoplasms, leukemia and pernicious anemia resulted in an inhibitory effect on bone marrow cultures [56]. Given the results of these previous reports [54–57] and our previous work that showed that 4-week DDDF induced an anti-tumorigenic proteome in healthy subjects [10], the suppressor effect of dry-fasted serum may be dose-dependent, and dose-response studies are needed.

In parallel to the reduction in cell proliferation/viability in HepG2 cells after treatment with dry-fasted serum, there were also significant changes in serum proteins in subjects with metabolic syndrome and MASLD at the end of 4-week DDDF compared with the serum proteins collected before 4-week DDDF. There was an average 279-fold increase in CD248 GP level at the end of 4-week DDDF compared with CD248 GP levels before 4-week DDDF. CD248, also known as endosialin and tumor endothelial marker 1 (TEM1) plays a significant role in hepatocellular tumorigenesis and physiological and pathological angiogenesis [58–60]. Mogler et al. [58] demonstrated that endosialin, expressed by hepatic stellate cells, plays a critical role in suppressing the proliferation of hepatocellular carcinoma. Silencing endosialin in these cells decreases their proliferation and accelerates the progression of hepatocellular carcinoma [58]. The study also found that endosialin-deficient mice exhibited higher levels of hepatic tumor cell proliferation and tumor burden than wild-type mice [58]. These findings strongly support the interpretation that 4-week DDDF-induced CD248 upregulation in subjects with metabolic syndrome and MASLD may represent an immune-regulatory response, contributing to the suppression of HepG2 liver cancer cell proliferation observed in our study. Although the upregulation of CD248 was associated with tumor angiogenesis and metastasis [59], the upregulation of CD248 in the dry fasted serum at the end of 4-week DDDF is likely due to physiological angiogenesis rather than pathological tumor angiogenesis observed in several cancers. In fact, it was shown that CD248, located in muscle pericytes, was crucial in sprouting angiogenesis during skeletal muscle remodeling, and CD248 knockout mice failed to have capillary sprouting [60]. These findings align with the findings of our previous study that showed upregulation of tropomyosin 4 one week after 30-day DDDF in healthy subjects [10]. Tropomyosin 4 was shown to have a significant role in skeletal muscle remodeling [61]. Further research is necessary to examine the effects of DDDF on skeletal muscle remodeling and development. This is particularly important because a TRE regimen that omitted breakfast and permitted study participants to consume water, black coffee and tea during the fasting period led to significant loss of lean mass [25].

Serum proteomic analysis showed that DPP4, also known as CD26, was upregulated at the end of 4-week DDDF. Upregulation of DDP4 is likely associated with the anti-tumorigenic effect of human dry-fasted serum. Patients with gastric cancer were shown to have significantly decreased serum CD26 (DDP4) levels compared to healthy controls [62]. The same study also showed that serum CD26 levels were lower in patients with HER2-positive tumors than those with HER2-negative tumors [62]. Similarly, serum CD26 levels in preoperative patients with colorectal carcinoma were shown to be significantly lower compared with the serum CD26 levels in healthy controls [63].

Using serum proteomic analysis, we found an average 2-fold increase in LYVE1 and a 3-fold increase in LRP1 GP levels at the end of 4-week DDDF compared with the GP levels before 4-week DDDF. LYVE-1, a scavenger hyaluronan receptor and CD44 homolog, is located in the lymph vessels and hepatic sinusoids [64]. Patients with metastatic lung cancer were shown to have lower serum LYVE-1 levels compared with patients with non-metastatic lung cancer, and the size of the primary tumor showed an inverse correlation with serum LYVE-1 levels [65]. The expression of LYVE-1 was also shown to be downregulated in cirrhosis and hepatocellular carcinoma [64, 66]; the downregulation of LYVE-1, the scavenger hyaluronan receptor, likely resulting in overexpression of

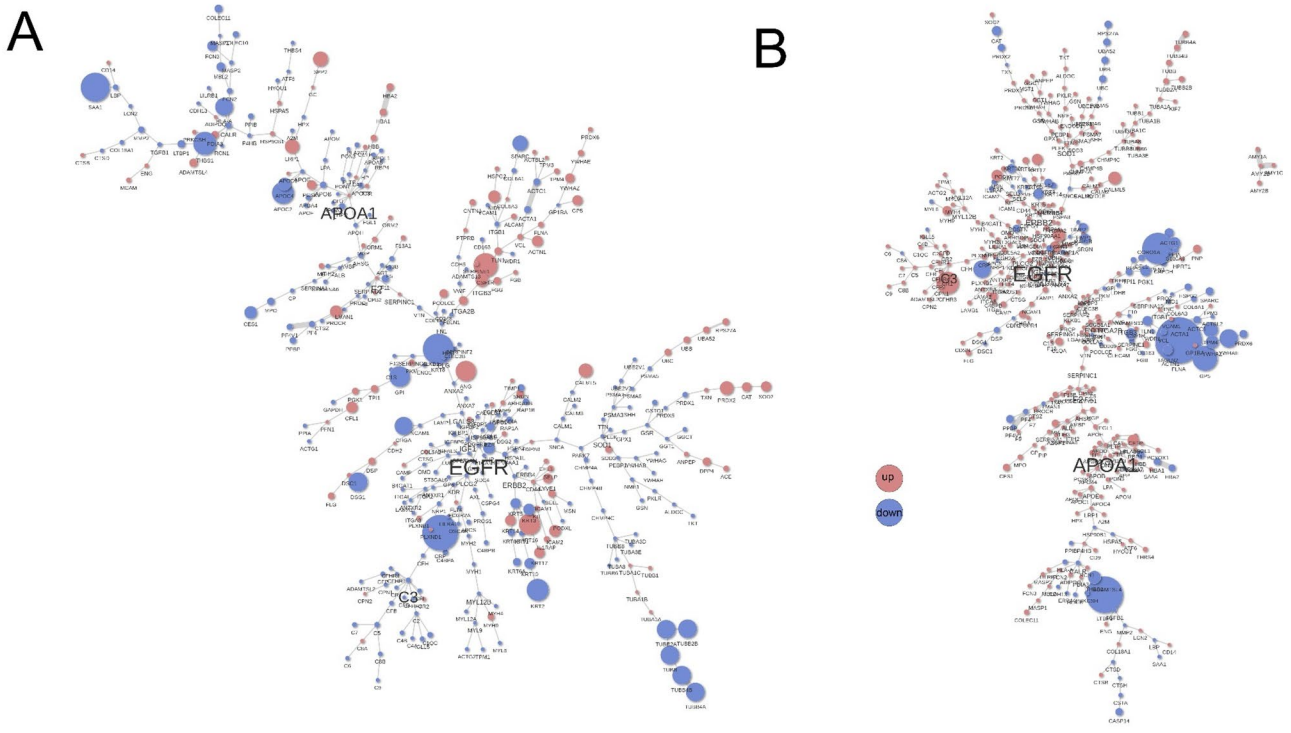

Fig. 4 Prize-Collecting Steiner Forest (PCSF) algorithm analysis of serum gene protein products (GP) relative aduncity change in (A) subjects with metabolic syndrome and metabolic dysfunction-associated steatotic liver disease (MASLD) and (B) healthy subjects at the end of 4-week dawn-to-dusk dry fasting (DDDF) compared with the GP levels before 4-week DDDF. The resulting PCSF subnetwork revealed that proteins associated with the EGFR and APOA1 pathways were generally decreased in serum samples collected from subjects with metabolic syndrome and MASLD at the end of 4-week DDDF, compared with serum samples collecteed before 4-week DDDF (A). In contrast, EGFR and APOA1 node protein levels were elevated in dry-fasted serum compared with non-fasted serum from healthy individuals (B)

hyaluronan and nuclear translocation of CD44-mediated pyruvate kinase M2 and thereby leading to the progression and poor prognosis in hepatocellular carcinoma [64, 66, 67]. Hyaluronan was shown to induce HepG2 liver cancer cell proliferation [67], which could also be related to the downregulation of LYVE-1. Similar to LYVE1, downregulation of LRP1 was also associated with invasiveness and poor prognosis in hepatocellular carcinoma [68]. Our results showing the upregulation of LYVE-1 and LRP1 in the serum after 4-week DDDF and reduction in HepG2 cell proliferation after treatment with dry-fasted serum align with the findings of these previous studies.

There are several human studies that reported anti-inflammatory benefits with dry fasting [69–72]. There are also murine studies that showed anti-tumorigenic effect with water fasting [9, 13]. Water fasting cycles lasting 48 to 60 h have been shown to delay tumor progression and enhance sensitivity to chemotherapy drugs in murine models of melanoma, neuroblastoma, and breast cancer [9]. In a murine osteosarcoma model, food deprivation during the active period produced a more significant anti-tumor effect compared to food deprivation during the inactive period and ad libitum feeding, and these outcomes were attributed to an improvement in the host's ability to control the tumor or to alterations in the tumor's circadian clock, or both [13].

The analysis of the PCSF subnetwork showed that proteins associated with the EGFR and APOA1 pathways were generally decreased in serum samples taken from subjects with metabolic syndrome and MASLD at the end of 4-week DDDF compared with the serum collected before 4-week DDDF (Fig. 4A). In contrast, EGFR and APOA1 node protein levels were elevated in serum samples taken from healthy subjects at the end of 4-week DDDF compared with the serum collected before 4-week DDDF (Fig. 4B). While the relationship between circulating EGFR and APOA1 levels and tumor cell growth remains complex and controversial, this differential alteration in EGFR and APOA1 levels may be associated with the observed inhibition of proliferation in the cultured HepG2 cells treated with these serum samples. For example, EGFR knockdown has been shown to reduce the proliferation and migration of HepG2 cells via Akt/GSK-3β/Snail signaling pathway [73]. The EGFR inhibition by erlotinib was found to overcome lenvatinib drug resistance in hepatocellular carcinoma by blocking the activation of the EGFR–STAT3–ABCB1 signaling pathway

[74]. One particularly intriguing aspect is the observed downregulation of APOA1 in serum collected from subjects with metabolic syndrome and MASLD. The downregulation of APOA1 is linked to increased tumor progression in cancers such as basal-like breast cancer and hepatocellular carcinoma, whereas overexpression of APOA1 has shown anti-proliferative and pro-apoptotic effects [75–77]. These findings suggest that both EGFR and APOA1 play critical roles in maintaining proliferative potential. Further investigation is needed to clarify the relationship between serum-level alterations of these proteins and their effects on cancer cell proliferation.

To determine whether the effect of human non-fasted and dry-fasted serums differed from that of a fetal bovine serum on HepG2 cells, we treated HepG2 cells with both human serum and FBS. The influence of human serum on cell proliferation was significantly greater than that of FBS which served as a control under standard cell growth conditions. This effect was consistent regardless of whether the serum was derived from healthy individuals or those with metabolic syndrome and MASLD, as well as whether the subjects were non-fasted or dry-fasted. This suggests that human serum supplementation is much more favorable for the growth of human-derived HepG2 cells (Figs. 2 and 3). The behavioral differences between human and bovine serum in tissue cultures were previously reported [78, 79]. Our results suggest that human serum should be used instead of FBS in cell culture experiments to mimic human metabolism.

The limitations and challenges of our study are as follows: To our knowledge, this is the first study to test human serum obtained from subjects who underwent 4-week DDDF on HepG2 liver cancer cell viability. The MTT assay, a widely used and well-established method for assessing cell viability, was employed in this study to evaluate HepG2 cell proliferation. However, it primarily reflects mitochondrial activity rather than direct cell death, meaning that metabolic alterations could influence the observed effects independently of changes in cell viability.

Because there is a lack of prior research in this area, we were unable to determine the statistical power in advance; therefore, we classify our study as a pilot study. Additionally, because we collected samples from a limited number of subjects, we could not account for other factors such as age, gender, or lifestyle, which limits the scope of our research.

Based on an expected mean difference of 15% in cancer cell proliferation between serum samples collected before and at the end of 4-week DDDF, and with an estimated standard deviation of 20%, a two-sided paired t-test with 80% power and a significance level of 0.05 requires a minimum of 15 matched serum samples to detect a statistically significant effect. The sample size was estimated using G*Power 3.1, a validated and widely used statistical software for power analysis in biomedical research [80]. Despite recognizing the limitations of our data, this study serves as proof-of-concept. Further validation in larger, independent cohorts using antibody-based methods could enhance our findings.

Our study demonstrated that a 4-week DDDF alters the serum proteome, regardless of whether these changes are statistically significant at a P-value of < 0.05. There is a possibility that DDDF has initiated a series of complex molecular changes throughout the body. These changes may represent a continuum that is not adequately captured by evaluating serum samples at just a single time point after the fasting period concludes. To fully understand the anti-tumorigenic effects of DDDF, it may be necessary to conduct a more comprehensive analysis over multiple time intervals, allowing researchers to gain deeper insights into how these changes unfold and interact over time. Our observations are likely related to the Warburg effect that represents metabolic stress and the reprogramming of protein translation that could be fatal to malignant cells [14–16].

Furthermore, we only evaluated human non-fasted and dry-fasted serum in HepG2 cell cultures and did not conduct a dose-escalation study. Given these limitations, our preliminary results suggest that future research should address the following critical questions: (1) Does human dry-fasted serum have a similar suppressive effect on cancer cell lines other than HepG2? (2) Is the effect of human dry-fasted serum on suppressing liver tumor cell viability and invasiveness dose-dependent? (3) Does co-culturing liver cancer cell lines with human dry-fasted PBMC produce a more substantial inhibitory effect on cell viability and invasiveness than culturing liver cancer cell lines with dry-fasted serum alone? Addressing these questions may uncover new therapeutic implications for dry-fasted serum, plasma, or blood in cancer treatment, particularly since many cancer patients cannot undergo dry fasting due to chemotherapy-related side effects such as nausea, vomiting, and diarrhea.

There may be potential challenges in translating DDDF into clinical practice. One anticipated challenge is ensuring subject compliance with the DDDF regimen, similar to any type of fasting program. Participants must schedule their meals at dawn and dusk, abstaining from eating and drinking during the daytime to align their mealtimes, wake-up times, and sleep times with the natural cycle of dawn and dusk. This adjustment requires a strong commitment to self-discipline and willpower [81].

Additionally, because the fasting duration spans from dawn to dusk, it will vary from day to day and season to season. This variability is expected and is actually beneficial, as it can help precisely reset the circadian clock. In contrast, fasting with a predetermined, fixed number of

hours would not align with the natural times of dawn and dusk and could disrupt the circadian rhythm.

Conclusion

To our knowledge, this is the first study demonstrating dry-fasted serum collected from subjects with metabolic syndrome and MASLD reduced proliferation/viability of HepG2 liver cancer cells in vitro and showed changes in the proteome in vivo compared with non-fasted serum collected from the same subjects. Our findings suggest that 4-week DDDF might be an intervention to induce proteomic responses for the prevention and adjunct treatment of metabolic syndrome-induced cancers (e.g., liver, colorectal, pancreas, breast cancer).

Supplementary Information

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Additional file 1

Additional file 2

Additional file 3

Additional file 4

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Author contributions

A.L.M. formulated the study hypothesis and study concept, designed the study, drafted and wrote the manuscript, contributed with conducting the study, analyzing data, and critically reviewing and finalizing the manuscript. K.E.M. contributed with conducting the study and critically reviewing the manuscript. A.R.O. contributed with conducting the study, and critically reviewing the manuscript. M.M.A. contributed with conducting the study, and critically reviewing the manuscript. Z.R.C. contributed with conducting the study and critically reviewing the manuscript. P.K.J. contributed with conducting the study, and critically reviewing the manuscript. S.Y.J. contributed with performing MTT and proteomic analyses, analyzing data, and critically reviewing the manuscript.

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Data availability

Data Availability Statement: The raw data are not publicly available due to privacy and ethical restrictions.

Declarations

Competing interests

The authors declare no competing interests.

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